Mutation Screening of the *CDKN2A* Promoter in Melanoma Families

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Germline mutations of *CDKN2A*, at 9p21, are responsible for predisposition to melanoma in some families. However, evidence of linkage to 9p21 has been demonstrated in a significant proportion of kindreds with no detectable mutations in *CDKN2A*. It is possible that mutations in noncoding regions may be responsible for predisposition to melanoma in these families. We have analyzed approximately 1 kb of the *CDKN2A* promoter upstream of the start codon in an attempt to identify causal mutations in 107 melanoma families. Four sequence variants were detected. Two of these (A-191G and A-493T) did not segregate with disease and were present in a control population at a comparable frequency, indicating that they are unlikely to predispose to melanoma. The A-493T variant appeared to be in linkage disequilibrium with the previously described *CDKN2A* polymorphism Ala148Thr. The variant G-735A was detected in the control population, but segregation of this variant with melanoma within families could not be discounted. The fourth variant (G-34T), located in the 5′ UTR, creates an aberrant initiation codon. This variant appeared to segregate with melanoma and was not detected in a control population. G-34T has recently been identified in a subset of Canadian melanoma families and was concluded to be associated with predisposition to melanoma. The creation of an aberrant initiation site in the 5′ UTR may have an important role in carcinogenesis in a small percentage of families; however, mutations in the *CDKN2A* promoter appear to have a limited role in predisposition to melanoma. *Genes Chromosomes Cancer 28:45–57, 2000.*

INTRODUCTION

The retinoblastoma pathway appears to be of critical importance in melanoma carcinogenesis (Bartkova et al., 1996). The *CDKN2A* gene, at 9p21, codes for the CDKN2A protein, which inhibits CDK4/cyclin D phosphorylation of the retinoblastoma protein. Germline mutations in the coding region of *CDKN2A* and in *CDK4* on 12q have been shown to predispose to melanoma (Dracopoli et al., 1996; Hayward, 1996; Zuo et al., 1996; Foulkes et al., 1997; Liggett and Sidransky, 1998; Soufir et al., 1998). Approximately half of the melanoma kindreds investigated to date have shown evidence of linkage to chromosome band 9p21 (Cannon-Albright et al., 1992, 1994; Gruis et al., 1993; Goldstein et al., 1994; MacGeoch et al., 1994; Walker et al., 1994). However, germline mutations in the coding region of *CDKN2A* have only been detected in half of these 9p-linked families (Dracopoli and Fountain, 1996; Hayward, 1996), implying that approximately 25% of all melanoma families are linked to 9p21 but do not have detectable *CDKN2A* mutations. This could indicate that coding mutations of *CDKN2A* have been missed (Dracopoli and Fountain, 1996), or alternatively that other genes at 9p21 may be responsible for predisposition to melanoma in these families.

One candidate at 9p21 for genetic predisposition to melanoma is the alternative first exon of *CDKN2A*, exon 1β, located approximately 12 kb upstream of and centromeric to *CDKN2A* exon 1α (Mao et al., 1995; Stone et al., 1995). Exon 1β is spliced onto exon 2 of *CDKN2A*, but translation of the mRNA occurs in a different reading frame, resulting in the production of a protein unrelated to CDKN2A, p14ARF (the mouse homolog is known as p19ARF) (Bates et al., 1998). This alternative transcript was shown to be able to induce growth arrest in mammalian fibroblasts and was thought to play a similar role in cell cycle regulation to that of

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CDKN2A (Quelle et al., 1995; Liggett et al., 1996). Further, p14ARF acts as an MDM2 inhibitor enhancing *TP53*-related function (Bates et al., 1998). However, there has been no evidence to implicate p14ARF in melanoma carcinogenesis. Disease-associated mutations of this alternate transcript have not been detected independently of mutations affecting *CDKN2A* itself (FitzGerald et al., 1996; Quelle et al., 1997).

CDKN2B, which lies approximately 21 kb centromeric to *CDKN2A*, has also been implicated as a potential tumor suppressor gene at 9p21. Its product CDKN2B is another member of the INK4 family of cyclin inhibitors, with homology to CDKN2A. *CDKN2B* has been found to be deleted in conjunction with *CDKN2A* in a variety of tumor-derived cell lines (Gemma et al., 1996). However, once again no evidence of germline deletions or alterations of *CDKN2B* has been identified in melanoma families (FitzGerald et al., 1996; Liu et al., 1997; Walker et al., 1998).

Noncoding mutations are another possible cause of melanoma susceptibility in kindreds with 9p21 linkage. As methylation of the promoter region has been shown to play a role in tumorigenesis (Bird, 1992), we speculate that sequence alterations in this region might predispose to disease by reducing the level of transcription of the CDKN2A protein. We have tested the hypothesis that mutations in the promoter sequence of *CDKN2A* are responsible for defective function in a subset of melanoma families with no detectable *CDKN2A* coding mutations.

The region critical for promoter function has been shown to be contained within the 869 bases preceding the *CDKN2A* coding domain. The approximate positions of transcription initiation sites have been determined by RNase protection assay and disruption of these sites has been shown to completely abolish promoter activity (Hara et al., 1996). Germline mutations in critical regions of the *CDKN2A* promoter could reduce or abolish promoter function, resulting in a familial predisposition to disease.

We have previously reported the screening of 42 U.K. and six U.S. melanoma families for mutations in the coding region of *CDKN2A*, exon 2 of *CDK4*, and *CDKN2D*, another member of the INK4 family of cyclin inhibitors (Harland et al., 1997; Newton Bishop et al., 1999). Here we report the screening of the critical region of the *CDKN2A* promoter for mutations that may predispose to melanoma in the six U.S. families and 41 of the U.K. kindreds (DNA samples from U.K. family MEL 37 were not available for this study). An additional 30 Australian melanoma kindreds, previously screened for *CDKN2A* coding mutations (Holland et al., 1995, 1999; Mann et al., 1997) and 29 *CDKN2A* and exon 2 *CDK4* mutation negative Italian kindreds (Ciotti et al., 1996) have also been included in this study.

It is important to consider the significance of individual promoter variants with respect to their possible involvement in predisposition to melanoma. To do this, we examined the prevalence in the general population of any variant detected using a panel of 120 samples from normal controls. In addition, the segregation of each variant with disease within the families was investigated.

MATERIALS AND METHODS

Patients

Forty-one previously described U.K. melanoma families, recruited in the period since 1989, were screened for sequence changes in the *CDKN2A* promoter. In addition, 6 previously described U.S. families (Goldstein et al., 1994; Liu et al., 1997; Newton Bishop et al., 1999), 30 Australian melanoma families (Holland et al., 1995, 1999; Mann et al., 1997), and 29 Italian families (Ciotti et al., 1996) were also investigated. All three exons of *CDKN2A* and exon 2 of CDK4 had previously been investigated for mutation in each kindred. The U.K., U.S., and Australian samples (78 families in total) were screened by sequencing by M.H. The Italian samples (29 families) were screened by SSCP in the laboratory of G.B.-S. and P.G. Where possible, two or three melanoma cases were screened from each of the 107 families. However, in the majority of kindreds (78 families), only one melanoma case was available. For this reason, studies to assess the evidence for 9p linkage were not feasible, so all families were examined for promoter mutations. Ethics committees in all institutions approved the studies.

PCR Amplification and Sequencing of *CDKN2A* Promoter

Primers were designed to amplify a 1,185-bp fragment of DNA containing the entire region of the *CDKN2A* promoter shown to be essential for function (Hara et al., 1996). For the initial amplification, the forward primer, p16 from 2F (TCTGGTCTAGGAATTATGAC), was designed from the *CDKN2A* promoter sequence (GenBank accession number X94154) and lies at -949 to -930 relative to the first base of *CDKN2A*. The reverse primer, p16 ex 1R (GCGCTACCTGATTC-

TABLE 1. Sequencing Primers Used for Analysis of *CDKN2A* Promoter

Forward primers	Location[a]	Sequence	Reverse primers	Location[a]	Sequence
p16 prom 2F	−949 to −930	TCTGGTCTAGGAATTATGAC	p16 prom 1R	+49 to +30	CCAGCCAGTCAGCCGAAGGC
p16 prom 4F	−542 to −523	AGCCATACTTTCCCTATGAC	p16 prom 2R	−182 to −201	AGGGGACGCTGTGAGCGAGT
p16 prom 6F	−183 to −164	CTTGCCTGGAAAGATACCGC	p16 prom 3R	−482 to −501	TACAACCTTCCTAACTGCCA

[a]Location is given as position relative to A of first ATG of *CDKN2A* coding sequence (5′–3′).

CAATTC) lies in intron 1 of *CDKN2A* and has previously been used in the amplification of exon 1 of *CDKN2A* (Harland et al., 1997). Fifty-μl PCR reactions were carried out using 100-ng genomic DNA, 0.2-mM dNTPs, 50 μM each primer, 10% dimethyl sulphoxide (DMSO), and 2 units of DeepVent DNA Polymerase (New England Biolabs) in the reaction buffer provided by the supplier. Thermal cycling consisted of 30 cycles of denaturing at 94°C (30 sec), annealing at 55°C (30 sec), and extension at 72°C (90 sec), with a final 7-min extension at 72°C. PCR fragments were isolated by gel electrophoresis and purified prior to sequencing using the QIAquick Gel Extraction Kit (Quiagen).

Sequencing reactions were carried out using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin Elmer) and the products were analyzed using an ABI 377 DNA Sequencer. To ensure complete coverage of the area of *CDKN2A* promoter essential for function, sequencing reactions were initiated from three primers in the forward direction and also three primers in the reverse direction. The sequencing primers used are listed in Table 1.

SSCP Analysis of *CDKN2A* Promoter

Primers were designed to obtain useful SSCP fragments from approximately 1,200 bp of the *CDKN2A* promoter region (Table 2), which was divided in five regions of amplification. Twenty μl PCR reactions were carried out using 100-ng genomic DNA, 0.2-mM dNTPs, 20 mM each primer, 2.5-M betaine, and 0.5 units of AmpliTaq DNA Polymerase (Perkin Elmer) in the reaction buffer provided by the supplier. Thermal cycling consisted of 5′ initial denaturation at 94°C followed by 30 cycles of denaturing at 94°C (30 sec), annealing at 55°C (1 min), and extension at 72°C (40 sec), with a final 5′ extension at 72°C. SSCP on gradient polyacrylamide gels (from 5% to 20%) were run for 14 hr at 400 V on a horizontal electrophoresis apparatus (Multiphor, Pharmacia) at two different temperatures (12°C and 23°C). Single strands were visualized using silver staining. As a quality control measure, examples of each sequencing-detected variant identified in the U.K., Australian, or U.S. samples were sent to the laboratory of G.B.-S. and P.D. to confirm that SSCP identified the sequence changes. All sequencing-detected variants were identified clearly by SSCP analysis.

The same primers used for amplification were used for direct sequencing of variants and controls, after purification of the samples by Quiagen-quick spin columns. Sequencing reactions were carried out using the ABI PRISM dRhodamine Terminator Cycle sequencing kit (Perkin Elmer), and the products were analyzed using an ABI 310 DNA Sequencer.

Analysis of Variants

We had multiple samples from affecteds in 29 kindreds. The segregation of any detected sequence variant with melanoma was investigated in informative families. In addition, the frequency of each variant in the general population was assessed using a panel of control DNA samples consisting of 100 controls with no history of cancer, collected in epidemiological case/control studies, and 20 samples obtained from healthy staff of ICRF Leeds. Differences in frequencies of variants in cases and controls were assessed using the appropriate chi-square test. For each of the biallelic polymorphisms, adherence to Hardy-Weinberg equilibrium was assessed with a 1-df chi-square test.

Restriction Digestion Tests for Variants at -191 and -735

To enable rapid and cost-effective analysis of the control population, restriction tests were designed for the detection of each variant where possible. The restriction enzyme *Acy*I was found to cleave the 1,185-bp *CDKN2A* promoter PCR fragment, producing bands of 760 bp and 425 bp if the mu-

TABLE 2. Primers Used for SSCP Analysis of *CDKN2A* Promoter

Forward primers	Location[a]	Sequence	Reverse primers	Location[a]	Sequence
prom 1F	−1189 to −1168	GCTTAGGATGTGTGGCACTGT	prom 1R	−908 to −890	AGGATCCGAATGGGGAGGGAGTCATTGG
prom 2F	−931 to −912	AGGATCCGTTTGAGAATGGAGTCCGTCC	prom 2R	−662 to −640	CCCTACCCCCTCAACCCTTG
prom 3F	−684 to −667	AGGATCCGTAGTGAACCCCGCGCTCCTG	prom 3R	−417 to −397	CCCCCGCCTGCCAGCAAAGGC
prom 4F	−436 to −418	AGTGAACGCACTCAAACACG	prom 4R	−157 to −138	AGGATCCGTGTCCCTCAAATCCTCTGGA
prom 5F	−240 to −220	TTCGCTAAGTGCTCGGAGTT	prom 5R	−5 to +17	CGGGATCCCGCCGCCGGCTCCATGCTGC

[a]Location is given as position relative to A of first ATG of *CDKN2A* coding sequence (5′–3′).

tant G-allele was present at -191, but not if the wild-type A-allele was present. *Ksp*632I was found to cleave the wild-type *CDKN2A* promoter PCR product once, creating two fragments (354 bp and 831 bp); however, if the mutant A-allele was present at -735, the 1,185-bp PCR product was cleaved twice, producing bands of 612 bp, 354 bp, and 219 bp. Restriction digests were carried out in 20-μl reactions; 10 μl of PCR product was digested using 5 units of restriction enzyme at 37°C for 2 hr. Sequencing and restriction data were initially cross-checked to ensure the validity of the restriction tests.

PCR Tests for Variants at -34 and -493

Restriction tests could not be designed to detect the G-to-T variant at -34, or the A-to-T variant at -493, as restriction sites are neither created nor destroyed at these sites. A PCR test was designed for these variants using wild-type and mutation-specific reverse primers and a common forward primer. The mutation-specific primer only amplifies the mutant allele, and the wild-type allele is amplified exclusively by the wild-type–specific primer (Liu et al., 1999). Primers were designed using the published *CDKN2A* promoter sequence (GenBank accession number X94154) and are listed in Table 3. Twenty-five μl of PCR reactions were carried out using 25-ng genomic DNA, 0.2-mM dNTPs, 50 mM each primer, 10% DMSO, 1.5-mM $MgC1_2$, and 1 unit of AmpliTaq Gold DNA polymerase (Perkin Elmer) in the reaction buffer supplied by the manufacturer. Thermal cycling consisted of an initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturing at 94°C (30 sec) annealing at 61°C (30 sec) and extension at 72°C (1 min) with a final 7-min extension at 72°C. Sequencing and PCR data were initially cross-checked to ensure the validity of the PCR tests.

RESULTS

Minor differences were observed between our *CDKN2A* promoter consensus sequence and the sequence listed in GenBank (accession number X94154). These included two thymine residues, which were present in the GenBank sequence at -605 and -628, but were not observed in any of the samples sequenced in this study. For this report, the *CDKN2A* promoter has been numbered according to the corrected sequence. Numbering is relative to the first base of the *CDKN2A* start codon, so that the first base of the start codon is numbered +1, and the base immediately preceding it is numbered -1. Two other differences observed were at

TABLE 3. Primers Used in PCR Tests for *CDKN2A* Promoter Variants at −34 and −191

−34 G-to-T	Primer	Location[a]	Note	−493 A-to-T	Primer	Location[a]	Note
p16 pro F	GACTTAGTGAACCCCGC GCTCCTGA	−686 to −662	Forward primer[b]	p16 pro F	GACTTAGTGAACCCCGC GCTCCTGA	−686 to −662	Forward primer
p16 F	GGCTCTTCCGCCAGCAC CG	−126 to −108	Forward primer[c]	−493 Mut-R	CCTCCGCGATACAACCT TCCA	−473 to −493	Reverse, mutation-specific
−34 Mut-R	GCGCGCTGCCTGCTCTC CCCA	−14 to −34	Reverse, mutation-specific	−493 WT-R	CCTCCGCGATACAACCT TCCT	−473 to −493	Reverse, WT-specific
−34 WT-R	GCGCGCTGCCTGCTCTC CCCC	−14 to −34	Reverse, WT-specific				

[a]Location is given as position relative to A of first ATG of *CDKN2A* coding sequence (5′–3′).
[b]In the laboratory of J.N.B. and M.H., UK.
[c]In the laboratory of G.M. and E.H., Australia.

residue –30 (G rather than T) and residue –5 (C rather than A). Four different variants were detected in the 1-kb region of promoter sequenced (Tables 4 and 5). These were G-to-A at nucleotide -735 (G-735A), A-to-T at -493 (A-493T), A-to-G at -191 (A-191G), and G-to-T at -34 (G-34T). Examples of variants detected by sequencing and SSCP are shown in Figure 1.

A-191G

A-191G appeared to be a common polymorphism and the G-allele was present homozygously in many cases. Where two melanoma cases from a single family were screened, the G/A status at -191 for both individuals is shown (Tables 4 and 5). Fifty-two of the 135 (39%) cases screened were found to be homozygous wild-type, 61 cases (45%) were heterozygous A/G, and 22 cases (16%) were homozygous for G ($P > 0.8$ for test of deviation from Hardy-Weinberg equilibrium, HWE). Of the 120 control DNA samples, 46 (38%) were found to be homozygous wild-type A/A, 57 (48%) were found to be heterozygous A/G, and 17 (14%) were found to be homozygous G/G by *Acy*I restriction digest ($P = 1.0$ for deviation from HWE; chi-square = 0.26 with 2 df, $P = 0.88$ for equality with case distribution; Fig. 2). The -191 variant was found not to segregate with disease in the six U.K. melanoma kindreds in which it could be studied (Fig. 3).

G-735A

G-735A was found to be present in members of 6 of the 107 families studied. A total of 6 individuals from 135 melanoma cases screened were heterozygous for this variant (4%), as were 6/120 (5%, $P > 0.8$ for differences between cases and controls) control samples screened by *Ksp*632I digestion (Fig. 2). However, segregation of this variant with melanoma could not be completely ruled out in the two pedigrees in which it could be studied (Fig. 3).

A-493T

The variant A-493T was observed in some members of seven families. A total of nine individuals from 135 melanoma cases screened were heterozygous for this variant (7%), as were 7/120 (6%) control samples ($P > 0.8$ for difference between cases and controls) analyzed by mutation-specific PCR (Fig. 2). The variant A-493T did not segregate with disease in the kindreds investigated (Fig. 3).

TABLE 4. Summary of Melanoma Kindreds by Number of Cases of Melanoma and Showing Sequence Variants Identified

Pedigree number[a]	Number of cases	*CDKN2A* exon 1[b]	*CDKN2A* exon 2[b]	*CDKN2A* promoter −191 A/G[c]	*CDKN2A* promoter other variants[d]
31220	13	Arg24Pro		A/A	
MEL21	10	Arg24Pro		A/G	
NIH 1	10		*Ala148Thr*	A/G	−493 A-to-T
20146	8			G/G	−735 G-to-A
20963	7		*Ala148Thr*	A/G, A/G	−493 A-to-T
42938	7			G/G	−735 G-to-A
21266	6			A/A	
21273	6			A/A, A/G	
21568	6			A/A	
22070	6			A/A	
20012	5			A/G	
21065	5			A/G	
21128	5	Ala36Pro		A/A	
21194	5			A/A	
21519	5		Met53Ile	A/G	
21566	5			G/G	−34 G-to-T
31662	5			G/G	
MEL07	5			A/G	
MEL09	5		Met53Ile	A/A	
MEL32	5		Met53Ile	A/G	
MEL48	5			A/G	
NIH 2	5			A/G	
20008	4		*Ala148Thr*	A/A, A/G	−493 A-to-T
20176	4			A/G	
20442	4			A/A	
20696	4		*Ala148Thr*	A/G	−493 A-to-T
20878	4			A/G	
21166	4			A/G	
21167	4	Ile49Ser		A/G	−735 G-to-A
21506	4			A/G	
21509	4			A/G	
21617	4			A/A	
21670	4			G/G	
21711	4	Trp15Stop		A/G	
21971	4			A/G	
31666	4		Arg112Gly	A/A	
MEL02	4			A/G	−735 G-to-A
MEL03	4			A/A, A/A	
MEL06	4			A/A	
MEL13	4	23ins24		A/A, A/A	
MEL15	4		Ala118Thr	A/A	
MEL26	4			A/A, A/A	
MEL39	4		Gly67Arg	A/A, A/G	
MEL40	4	Arg24Pro		A/G	
MEL49	4			A/G	
20050	3		*Ala148Thr*	G/G, G/G	−493 A-to-T
FMM3	3			A/G, G/G	
FMM5	3		*Ala148Thr*	A/G, G/G	−493 A-to-T
FMM7	3			A/A, A/G	
FMM24	3			A/A, A/G	
FMM29	3			G/G	
MEL24	3	88delG		A/A, A/G	
MEL28	3			A/G, A/G	

TABLE 4. Continued

Pedigree number[a]	Number of cases	*CDKN2A* exon 1[b]	*CDKN2A* exon 2[b]	*CDKN2A* promoter −191 A/G[c]	*CDKN2A* promoter other variants[d]
MEL30	3			A/G,A/G	
MEL35	3			A/G	−735G-to-A
MEL44	3			A/A,A/G	
MEL50	3			A/G	
MEL53	3			A/A,A/A	
NIH 3	3		Asn71Ser	A/G	
NIH 4	3			A/G	

[a]Kindreds are listed in order of number of affected individuals in each family. The table lists kindreds with three or more cases of melanoma (the generally accepted criteria for a melanoma family). Those kindreds prefixed with "MEL" are U.K. families; "NIH" are U.S. families; "FMM" are Italian families; the remainder are Australian families.
[b]Mutations detected in exon 1 and 2 of *CDKN2A* are shown for each family; polymorphisms are shown in italics. Only positive findings are indicated. *CDKN2A* exon 3 and *CDK4* exon 2 were also screened in each kindred and found to be wild-type.
[c]A separate column indicates the A/G status at −191 in the *CDKN2A* promoter for each case tested.
[d]The presence of the three remaining sequence variants (G-735A, A-493T, and G-34T) is shown.

Linkage Disequilibrium Between A-493T and Ala148Thr

A total of 137 melanoma cases were screened for the A-493T promoter variant and 10 cases (7%) were found to carry the variant. It was noted that, in a separate analysis, exactly the same set of 10 cases was found to carry the Ala148Thr coding variant. This association is highly significant ($P < 0.0000001$ using Fisher's exact test). Among the 120 control samples, 7 were found to have both the variants while 113 had neither ($P < 0.0000001$ using Fisher's exact test). Evidence that the two variants are located within the same haplotype comes from examination of a family segregating the variants.

Analysis of the frequency of the haplotype bearing the A-493T promoter variant and the Ala148Thr coding variant showed no difference between the prevalence in cases as compared to controls (Yates corrected chi-square = 0.31, $P = 0.58$).

G-34T

G-to-T at nucleotide -34 was detected in 1 of the 107 (0.9%) families screened (Australian family number 21566). The G-34T promoter variant was found to segregate with disease in this kindred (Fig. 3). This variant was not detected in any of the 120 control samples investigated using the G-34T mutation-specific PCR test (Fig. 2). A further 103 *CDKN2A*-negative Australian probands (38 from three-case kindreds and 65 from two-case clusters) were analyzed by a PCR assay specific for the -34 G-to-T variant in the laboratory of G.M. and E.H. Only one further carrier was found, a proband from a two-case sibship. Other family members were not available for study.

DISCUSSION

There is a need for greater understanding of predisposition to melanoma given that more families show evidence of 9p21 linkage than have *CDKN2A* coding mutations (Dracopoli and Fountain, 1996; Hayward, 1996). *CDKN2A* is a relatively small gene, which has allowed a thorough investigation of the coding region, and as a result it is unlikely that coding mutations in *CDKN2A* have gone undetected. It is therefore possible that there may be other determinants of predisposition at this location.

The issue of melanoma families linked to 9p21 with no detectable *CDKN2A* mutations is mirrored in other, larger, tumor suppressor genes. For example, screening has failed to identify *BRCA1* mutations in a proportion of breast cancer families with evidence of linkage to chromosome arm 17q (Ford et al., 1998).

Other genes encoded in the same cluster as *CDKN2A* (*P14ARF*, *CDKN2B*) have been investigated in an attempt to identify an alternative 9p21 tumor suppressor gene. However, evidence for the involvement of these genes has been limited, with few examples of deletion or alteration independent of *CDKN2A*. None has been found to be mutated in melanoma kindreds (FitzGerald et al., 1996; Liu et al., 1997; Quelle et al., 1997; Kumar et al., 1998).

Attention has increasingly turned to the investigation of the control sequences of tumor suppressor genes. Methylation of CpG islands in the promoter region of tumor suppressor genes has been shown to be an important mechanism of transcriptional repression/silencing and may play an important role in tumorigenesis (Bird, 1992). In *CDKN2A*, meth-

TABLE 5. Summary of Melanoma Kindreds by Number of Cases of Melanoma and Showing Sequence Variants Identified

Pedigree number[a]	Number of cases[e]	*CDKN2A* exon 1[b]	*CDKN2A* exon 2[b]	*CDKN2A* promoter −191 A/G[c]	*CDKN2A* promoter other variants[d]
MEL01	2 (1st)			A/G, G/G	
MEL04	2 (2nd)			A/A	
MEL11	2 (1st)		*Ala148Thr*	A/A, A/G	−493 A-to-T
MEL14	2 (1st)			G/G	
MEL16	2 (1st)			A/A	
MEL19	2 (1st)			A/G	
MEL20	2 (2nd)			A/A, A/A	
MEL22	2 (1st)			G/G	
MEL23	2 (1st)			G/G, G/G	
MEL25	2 (2nd)			A/G, G/G	
MEL29	2 (1st)	23ins24		A/A, A/A	
MEL34	2 (1st)			G/G	
MEL38	2 (1st)			A/A	
MEL41	2 (2nd)			A/G	
MEL42	2 (1st)			A/A, A/G	
MEL43	2 (1st)			A/G	
MEL46	2 (1st)			A/A	
MEL54	2 (1st)			A/G	
MEL55	2 (1st)			A/G	
MEL56	2 (1st)			A/G	
NIH 5	2 (1st)			A/G	
NIH 6	2 (1st)			A/A	
FMM1	2 (1st)			A/G	
FMM2	2 (1st)			G/G	
FMM4	2 (1st)		*Ala148Thr*	A/A, A/G, G/G	−493 A-to-T
FMM6	2 (1st)			A/A	
FMM8	2 (2nd)			A/A	
FMM9	2 (2nd)			A/A	
FMM10	2 (2nd)			A/A, A/G	
FMM11	2 (2nd)			A/A, G/G	
FMM12	2 (1st)			A/A	
FMM13	2 (1st)			A/G	
FMM14	2 (1st)			G/G	
FMM15	2 (1st)			A/A, A/G	
FMM16	2 (1st)			A/A	
FMM17	2 (1st)			A/G	
FMM18	2 (1st)			A/A, A/G	
FMM19	2 (1st)			A/G	−735 G-to-A
FMM20	2 (1st)			A/A	
FMM21	2 (2nd)			A/G	
FMM22	2 (1st)			A/A	
FMM23	2 (1st)			A/G	
FMM25	2 (1st)		*Ala148Thr*	G/G	−493 A-to-T
FMM26	2 (1st)			A/A	
FMM27	2 (1st)			A/A	
FMM28	2 (1st)			A/A	

[a]Kindreds are listed in order of number of affected individuals in each family. The table lists kindreds with two cases of melanoma. Those kindreds prefixed with "MEL" are U.K. families; "NIH" are U.S. families; "FMM" are Italian families; the remainder are Australian families.
[b]Mutations detected in exon 1 and 2 of *CDKN2A* are shown for each family, polymorphisms are shown in italics. Only positive findings are indicated. *CDKN2A* exon 3 and *CDK4* exon 2 were also screened in each kindred and found to be wild-type.
[c]A separate column indicates the A/G status at −191 in the *CDKN2A* promoter for each case tested.
[d]The presence of the three remaining sequence variants (G-735A, A-493T, and G-34T) is shown.
[e]The degree of relatedness between the affected members of the two-case families is indicated in parentheses.

ylation of CpG islands has been associated with complete transcriptional block in a number of cancers, including brain, esophagus, lung, breast, and bladder (Herman et al., 1995; Merlo et al., 1995; Shapiro et al., 1995a, 1995b; Costello et al., 1996; Maesawa et al., 1996).

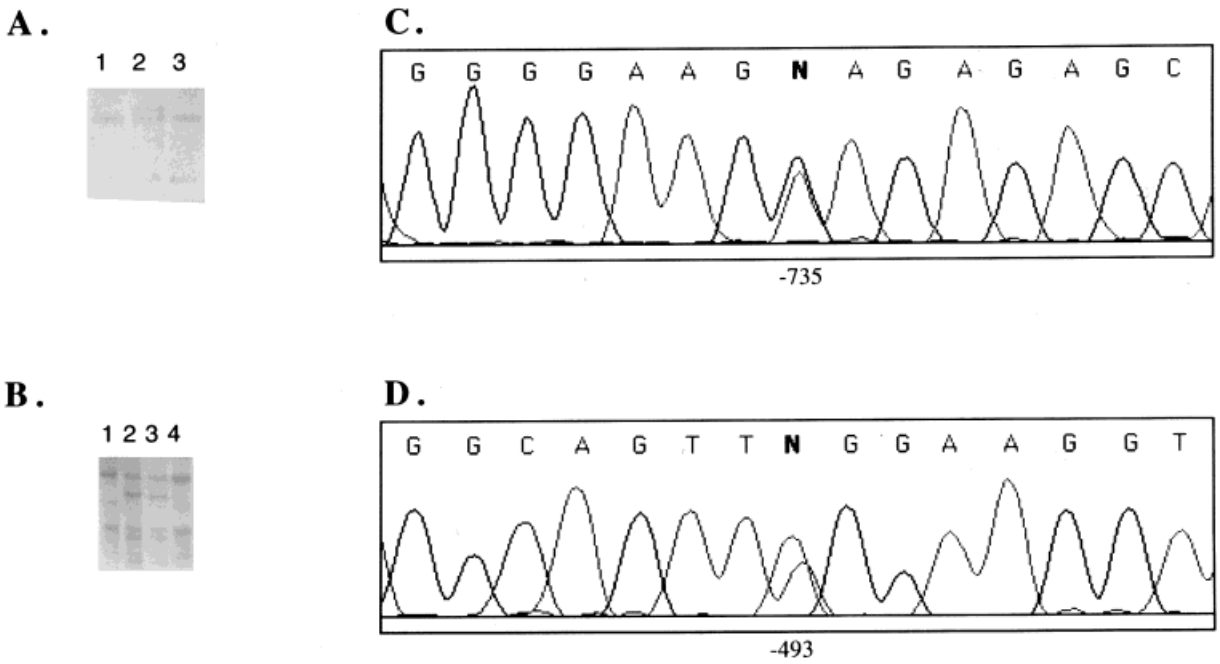

Figure 1. Examples of variants identified by SSCP by sequencing. **A:** G-735A identified by SSCP. Lanes 1 and 3 are wild-type; lane 2 is the variant. **B:** A-493T identified by SSCP. Lanes 1 and 4 are wild-type; lanes 2 and 3 are variants. **C:** G-735A identified by sequencing using the primer p16 prom 2R. **D:** A-493T identified by sequencing, using the primer p16 prom 2F.

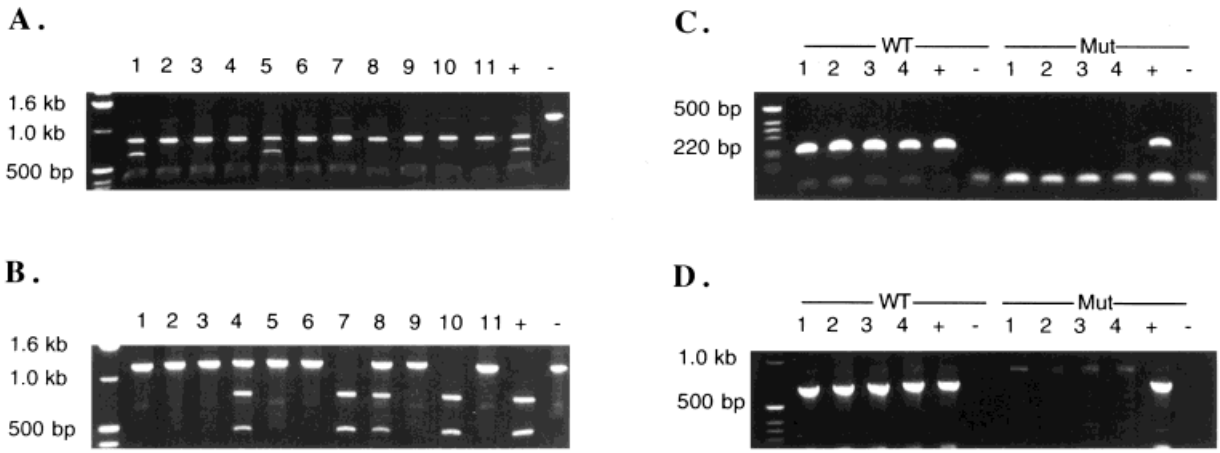

Figure 2. Examples of rapid screening for *CDKN2A* promoter variants. **A:** *Ksp*632I restriction digest test for G-735A. **B:** *Acy*I restriction digest test for A-191G. Lanes labeled 1–11 indicate control DNA samples taken from a melanoma case/control study. + and - denote positive and negative controls. **C:** Mutation-specific PCR test for A-493T. **D:** Mutation-specific PCR test for G-34T. Lanes labeled 1–4 indicate control DNA samples as above; + and - denote positive and negative controls. Lanes indicated by WT were amplified using wild-type–specific reverse primers; lanes indicated by Mut were amplified using mutation-specific reverse primer.

Sequence changes in a promoter have been shown in a number of cases to reduce the level of transcription of other genes resulting in familial disease. A 3-bp nucleotide deletion in the Sp1 binding site of the low-density lipoprotein receptor (LDLR) promoter resulted in a marked reduction in LDLR synthesis and has been implicated in familial hypercholesterolemia (Peeters et al., 1998). One form of congenital familial nonhemolytic hyperbilirubinemia is caused by a TA insertion at the TATA promoter region, which reduces the activity of the hepatic bilirubin UDP-glucoronosyltransferase gene (Clarke et al., 1997).

Previous work on *CDKN2A* regulation, using a reporter gene to study promoter activity in the region upstream of exon 1, has identified an 869-bp

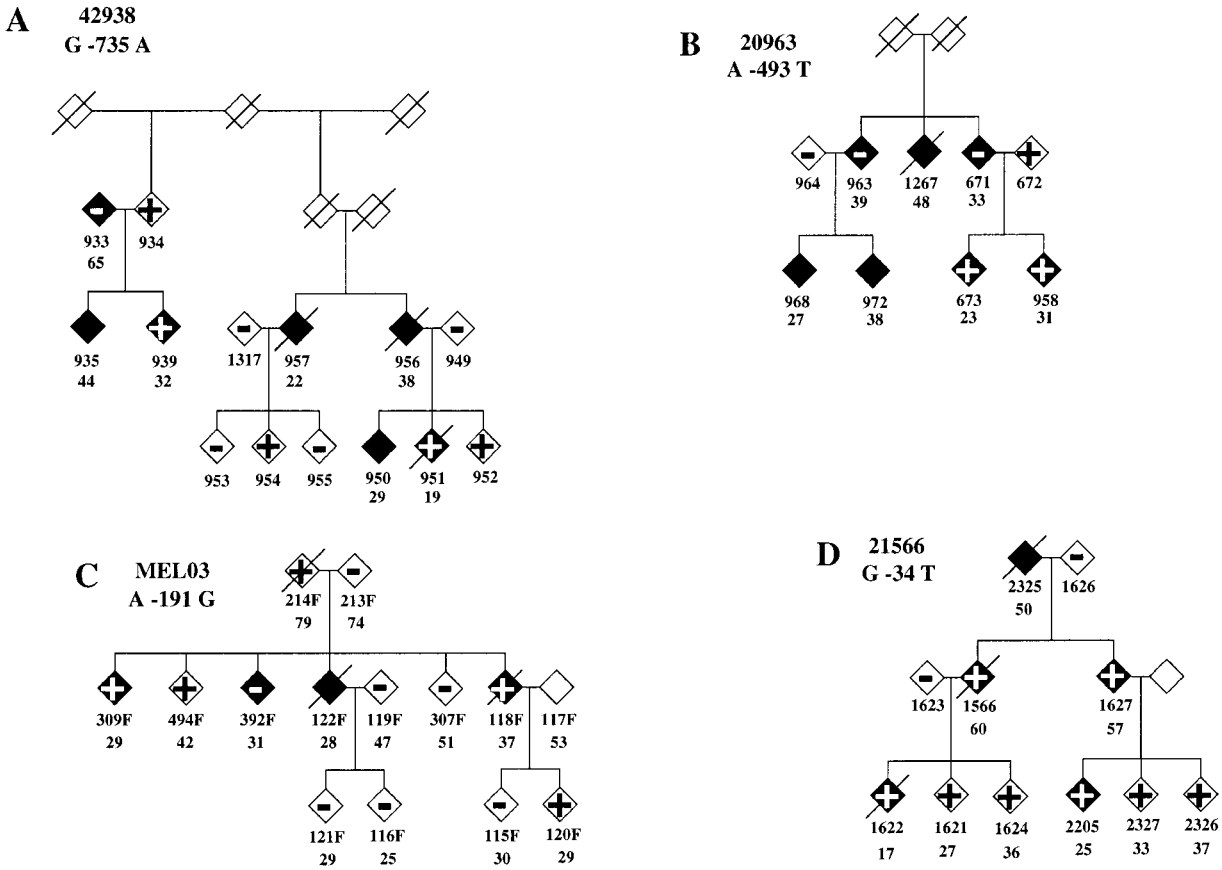

Figure 3. Segregation of *CDKN2A* promoter variants with disease in melanoma kindreds. Solid symbols indicate the presence of melanoma. + indicates carrier of variant; - indicates wild-type; other individuals not tested. Age at diagnosis of disease in affected and age at last contact in unaffected is shown below the individuals ID number. (**A**) G-735A variant segregates with melanoma, assuming that individual 933 represents a sporadic case. A-493T (**B**) and A-191G (**C**) variants do not segregate with melanoma. (**D**) G-34T segregates with melanoma.

fragment of DNA immediately 5′ to the start codon, which is essential for promoter function (Hara et al., 1996). This delineation of the vital region of the *CDKN2A* promoter has enabled an investigation into its potential role in tumorigenesis with the certainty of full coverage.

In this report we have analyzed 107 families, from the U.K., Australia, Italy, and the U.S., with evidence of predisposition to melanoma. Seventeen of the kindreds investigated carried germline mutations in the coding region of *CDKN2A*. Sequence analysis and SSCP has revealed four different variants in the *CDKN2A* promoter, at -34, -191, -493, and -735 (Tables 4 and 5). The use of two different screening techniques in two laboratories has ensured that variants are less likely to be missed in the analysis of the *CDKN2A* promoter.

The -34 G-to-T sequence variant could potentially create a false ATG methionine initiation site 35 bases upstream of the correct initiation site. The nucleotide sequence around this point matches the Kozak consensus sequence required for initiation of translation (Kozak, 1996). Initiation of translation from this false ATG would presumably result in a frameshift and truncation of the protein in exon 1. The -34 promoter variant has recently been reported in four Canadian melanoma kindreds. Flag epitope-tagged *CDKN2A* expression constructs, which enable the detection of full-length protein by Western blotting, were designed. Using these, wild-type and not mutant constructs were detected, confirming that translation from the mutant ATG results in truncation of the protein (Liu et al., 1999). As was also demonstrated in this study, the -34 variant was shown to segregate with disease in the Canadian families investigated and was not detected in a panel of control DNA. It was concluded that this *CDKN2A* variant was associated with a predisposition to melanoma (Liu et al., 1999).

The G-to-T variant was found to be present in 4 out of 59 (7%) Canadian *CDKN2A*-negative fami-

lies with two or more affected individuals (Liu et al., 1999), compared with 2 out of 193 (1%) *CDKN2A*-negative families investigated in this study. Combining these data gives a frequency of 6/252 (2%). It would therefore appear that although this mutant is likely to be associated with predisposition to melanoma in some families, it is only present at approximately the same frequency as any other of the germline *CDKN2A* mutations. This single promoter variant cannot therefore account for all examples of predisposition to melanoma.

The three other variants detected in the *CDKN2A* promoter were shown to be present in the control population at approximately the same percentage as in melanoma cases. A-191G and A-493T were shown not to segregate with disease, indicating that they are probably polymorphisms and are very unlikely to confer a high risk of melanoma.

The surprising observation that the A-493T promoter variant and the Ala148Thr *CDKN2A* polymorphism are in complete linkage disequilibrium does not have a straightforward explanation; in total, 17 of the samples screened in this study carried both variants while 257 carried neither. The high frequency of the implied haplotype (3% of all haplotypes) suggests that it is an ancient haplotype (M. Iles, personal communication; Kruglyak, 1999a, 1999b) and indeed it was found in individuals from Australia, Italy, U.K., and U.S. The two polymorphisms are approximately 6 kb apart, a distance in which recombination might be expected to occur in approximately 1 in every 16,000 meioses, assuming a genome-wide estimate of 1 cM being equal to 1,000,000 bp. Assuming that a single ancestral chromosome spontaneously arose carrying both of these mutations, the prevalence of the haplotype is not inconsistent with the lack of recombinant chromosomes (the age of the founder chromosome could be less than 5,000 generations) (Kruglyak, 1999b). However, it is difficult to rationalize how this single progenitor chromosome arose in the absence of evidence of chromosomes carrying only a single mutation. It should be noted that we did not find any evidence of an increased risk of melanoma in carriers of this haplotype; the interpretation of the observation seems therefore to relate more to population genetics issues than melanoma genetics. Indeed, a similar observation has been made for *BRCA1*, where the two most common haplotypes constructed from four amino acid substitutions differ from each other in three amino acids and are also not associated with differences in risk (Dunning et al., 1997).

Although G-735A was demonstrated to be present in the panel of control DNA, the possibility of segregation of this variant with disease could not be ruled out. Further work on the effect of this variant on CDKN2A protein expression and its segregation with disease in other melanoma families needs to be carried out.

There are few previous reports of promoter mutations that reduce the level of protein transcription. Those that have been reported tend to disrupt important regulatory regions in the promoter, such as the Sp1 binding site (or GC box), and the TATA box (Clarke et al., 1997; Peeters et al., 1998). A search of the *CDKN2A* promoter sequence for the consensus sequences of cis-acting promoter elements revealed several possible locations for GC and CAAT boxes, none of which were disrupted by the variants detected in this study. The absence of causal mutations in the 107 kindreds investigated suggests that germline alterations in the *CDKN2A* promoter sequence are not likely to be a significant cause of melanoma predisposition worldwide.

It is possible that there may be causal mutations in other flanking or regulatory *CDKN2A* sequences, or in other as yet unidentified genes at 9p21, which would account for disposition to melanoma in those families with evidence of linkage to 9p21 but with no detectable *CDKN2A* mutations. Evidence of a gene that modifies CDKN2A expression has been found in Dutch families by the analysis of shared haplotype and its apparent association with familial risk (van der Velden et al., 1999). It is also conceivable that in a small proportion of families hemizygous deletion of one or more exons of *CDKN2A*, which would not be detected by PCR, could be responsible for predisposition to disease, as found for instance for *BRCA1* by Puget et al. (1999).

Our results suggest that disease-associated germline mutations in the *CDKN2A* promoter are rare in melanoma families. Indeed, the one potential causal mutation detected is not a true promoter mutation, but actually a mutation in the 5′ untranslated region of the *CDKN2A* gene, which extends to nucleotide -40. The -34 mutation is clearly an important cause of melanoma predisposition in a small number of families and can be investigated quickly and cheaply using the mutation-specific PCR test (Liu et al., 1999). However, we believe that routine intensive screening for mutations in the remainder of the *CDKN2A* promoter of melanoma families has limited utility.

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